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Simultaneous Estimation of Aspirin, Atorvastatin Calcium and Clopidogrel Bisulphate in a Combined Dosage form by RP-HPLC

Pankaj Yadav[1*], Mohamad Taleuzzaman[2] and Pradeep Kumar[3]

[1]Pharmacy College Saifai, UPUMS Saifai, Etawah, (U.P.), India.
[2]Faculty of Pharmacy, Maulana Azad University, Jodhpur, 342802, Rajasthan, India.
[3]Ch Sughar Singh College of Pharmacy, Jaswantnagar, Etawah, (U.P.), India.

Authors' contributions

This work was carried out in collaboration among all authors. Author PY designed the study performed analysis and first draft. Authors MT and PY performed analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A rugged robust RP-HPLC method has been developed and validated for the simultaneous quantification of Aspirin (ASP), Atorvastatin calcium (ATO) and Clopidogrel bisulphate (CLO) in a combined dosage form. Optimized mobile phase composition of 25 mM KH_2PO_4: Methanol (20:80% v/v) pH 3.0 (adjusted with 20% o-phosphoric acid) isocratic mode with flow rate of 1 ml min^{-1}, detection at 230 nm. Employed Princeton SPHER C18 (150 x 4.6 mm i.d. 5 µm) column for separation at ambient temperature with an isocratic flow. The linearity of each drug across the range of 0.5-32 µg/ml with correlation coefficient for ASP, ATO, and CLO in value of 0.979, 0.988 and 0.989 respectively. The retention time (min.) for ASP, ATO and CLO were found 1.85, 3.04 and 6.91 respectively. Limit of detection (LOD) of ASP, ATO and CLO - 0.04, 0.04, 0.08 (µg ml^{-1}) respectively and Limit of Quantification (LOQ) of ASP, ATO and CLO- 0.14, 0.13, 0.28 (µg ml^{-1}) in order. Performed analysis of marketed tablet formulation ASP (Ecosprin-75 mg), ATO (Aztor®40-40 mg), CLO (Clopivas- 75 mg) found percentage recovery for ASP (99.93), ATO (100.00) and CLO

**Corresponding author: E-mail: pankajyadav0923@gmail.com;*

(99.87). Performed stability studies found satisfactory results. Developed method has been validated, results of different parameter found in acceptable range. Chromatogram peaks are resolve, symmetric shape and without interference.

Keywords: RP-HPLC; validation; LOD; LOQ; stability; peaks.

1. INTRODUCTION

Aspirin (ASP) IUPAC name (2-acetoxybenzoic acid) has molecular formula $C_{10}H_{12}O_4$ Fig. 1A is analgesic and antipyretic. It inhibits platelet aggregation by irreversible inhibition of platelet cyclooxygenase and thus inhibits the generation of thrombooxygenase A2 a powerful inducer of platelet aggregation and vasoconstriction. Atorvastatin calcium (ATO) IUPAC, calcium; (3R, 5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3, 5-dihydroxyheptanoate molecular formula have $C_{33}H_{35}FN_2O_5$ Fig. 1B. This drug blocks the production of cholesterol from the liver and increases hepatic uptake of low-density lipoprotein cholesterol, prevent the breakdown of acetyl co-enzyme to mevalonate by competitive mechanism because there is a similarity in structure with HMG-CoA reductase. Finally, the production of cholesterol reduces. Clopidogrel hydrogen sulfate (CLO), IUPAC name methyl (+)-(S) (chlorophenyl)-6,7-dihydrothieno [3,2-c] pyridine-5(4H)-acetate hydrogensulfate has molecular formula $C_{16}H_{16}ClNO_2S$ Fig. 1C, is a novel thienopyridine derivative that irreversibly blocks adenosine diphosphate (ADP) and is important in platelet aggregation [1] in recent years, special attention has been devoted to Hypolipidemic and Antiplatelet drugs. Therefore based on market survey and available combination hypolipidemic and antiplatelet drugs were selected to optimize a method and endorse an assay method to simultaneously quantification of the three drugs.

Analytical technique is a necessary part for analysis of raw materials that reach in the quality control, drug substances, formulation, also in research and development. The liquid chromatographic technique is one of the best for qualitative and quantitative analysis. Authenticate the new method by experimenting with different parameters like accuracy, precision, reproducibility, and robustness, after that the new method will be employ for the routine analysis [2,3].

Analysis of ASP and CLP in tablet dosage form done by High-performance liquid chromatographic a study of stress conditions based on ICH guideline [4]. Earlier developed a method and validated by HPLC and HPTLC for ASA and CLP [5,6]. Reported HPLC analytical method with UV detector for the study of degradation ASP and ATO [7]. RP-HPLC analytical method for the simultaneous estimation of ASP and CLO [8]. ASP quantification by liquid chromatography technique HPLC [9,10,11] and by (HPTLC) [12]. In pharmaceutical different dosage form estimation of ATO by HPLC [13,14,15,16,17]. Quantitative analysis of CLO by HPLC [18, 19,20] and by HPTLC, [21,22].

After the study of previous different analytical techniques for individual or in a combination of

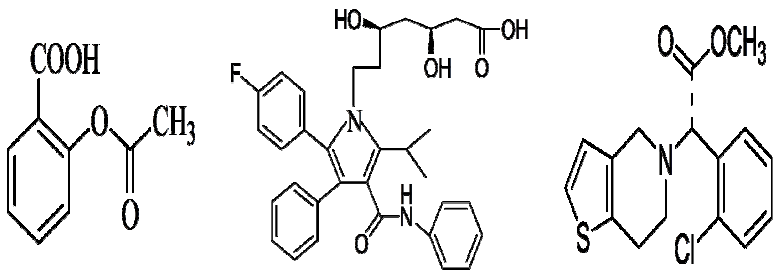

Figure 1A Figure 1B Figure 1C

Fig. 1. (1A,1B,1C). Chemical Structure of ASP, ATO and CLO

ASP, ATO and CLO. Attempt to further develop a method for the quantification in a combination of three drugs ASP, ATO and CLO in a pharmaceutical dosage form. Design an experiment for optimization of the mobile phase, pH, flow rate, and detection wavelength with effects on the retention time of the drugs.

1.1 Experimental

1.1.1 Chemical and Reagents

Methanol, Acetonitrile, Tetrahydrofuran, (Rankem Ltd., Delhi) O-Phosphoric acid (Merck Ltd., Mumbai), Triethylamine (Thomas Baker Ltd., Mumbai), Potassium dihydrogen orthophosphate (S. D. fine Chem. Ltd., Mumbai). *Marketed Formulation of Drug***-** For the estimation of Aspirin (Ecosprin-75 mg manufactured by USV), Atorvastatin Calcium (Aztor®40-40mg manufactured by Sun Pharma) and Clopidogrel Bisulphate (Clopivas-75 mg manufactured by Cipla) in pharmaceutical dosage forms- Tablets were used.

1.1.2 Instrumentation and chromatographic conditions

HPLC instrument employed for an experiment of company Shimadzu was furnished with a solvent delivery module LC-10 AT VP, Rheodyne injector 77251 used of volume capacity of 20 µL, and UV detector SPD 10A VP. Column RP C18 has a dimension (150 x 4.6 mm, 5 µ particle size). Mobile phase composition for the method optimized buffer (25 mM potassium dihydrogen phosphate): methanol (20:80). O-phosphoric acid was used to adjust the pH of the buffer to 3. Isocratic mode with a flow rate of the mobile phase was 1 mL min^{-1} and has a UV detector worked at 230 nm. Run time 10 minutes with injection volume 20 µL and retention time (min.) of the drug ASP-1.85, ATO-3.02, CLO-6.91. Spinchrome chromatographic station software was used for data acquisition. Optimized the mobile phase sonicate for 10 min. was found to be satisfactory for all these three drugs at 230 nm wavelength with the chromatographic condition.

1.1.3 Preparation of standard solution, calibration curve, and QC sample

Weigh carefully, 10 mg of each drug ASP; ATO and CLO and, transferred into separate 10 mL volumetric flask, liquefy in a minimum volume of acetonitrile. After that make up the volume 10 mL with solvent to get a 1000 µg mL^{-1} standard stock solution. Further, prepare a standard stock solution of strength 100 µg mL^{-1}. Then after, final dilution for calibration curve (0.5, 1, 2, 4, 8, 16 & 32 µg mL^{-1}) for working standard and mixed standard. Quality control standard solution, Low (0.75 µg mL^{-1}), Middle (3 µg mL^{-1}) and High (24 µg mL^{-1}).

1.1.4 Preparation of standard stock test solution

Weighed quantity equivalent to 10 mg and taken separately in a 10 mL volumetric flask. Solvate the solute in a minimum volume of acetonitrile, then after make up the volume 10 ml with acetonitrile. For proper mixing sonicate the solution for the 30 minutes. These solutions were centrifuged at 1000 rpm for 1 hour. 1ml of a supernatant solution of standard stock test solution of each solution was separately transferred into a 10 mL volumetric flask and added 9 mL of diluting solvent to get 100 µg mL^{-1}. Prepare six dilutions from stock solution (0.75, 1.5, 3, 6, 12, & 24 µg mL^{-1}).

2. METHOD VALIDATION

Validation of the developed method performed by conducting experiments as per the ICH guidelines. The parameters that need to performs experiment for the proper validation are-limits of detection (LOD), limits of quantification (LOQ), linearity range, precision, accuracy as recovery, robustness, and stability study [23,24].

2.1 Linearity

After optimized the final concentration, the calibration curve for all three drugs show a linear relationship of concentration directly proportional to the area of the peak. To establish the calibration curve performed three replicates (n=3). Concentration range for all three drugs 0.5- 32 µg mL^{-1} (ASP, ATO & CLO) made dilution from a stock solution.

2.2 Accuracy

To check the accuracy of the method experiment by injecting a fixed volume of sample (n=3) of known concentrations (3 µg) added three amounts in the percentage of 50, 100 and 150 in the fixed level of concentration. Calculate the value of SD and %RSD for all three drugs.

2.3 Precision

To conduct the precision determination of the method, analysis of the sample (n=6) of fixed concentration and determine the value standard deviation (SD) and percentage relative standard deviation (%RSD) of intra-day and inter-day analysis.

2.4 Recovery

Experiment performs by spiking the sample at three levels with 50%, 100% and 150% of standard solutions at a fixed concentration of individual drugs ASP, ATO, and CLO. Analyze the sample by the developed method and estimate the value of recovery in percentage.

2.5 Selectivity

Produce a chromatogram of the mobile phase then compare it with the chromatogram of a mixture of ASP, ATO & CLO tablet formulation. Check the interference at the retention time of individual drugs.

2.6 Limit of Quantitation (LOQ) and Limit of Detection (LOD)

Determine the value of both from the calibration curve. Find out the slope of calibration (s) and standard deviation of response (σ), then use formula k (σ/s) to calculate the value, where k for LOD is 3 and for LOQ its value 10.

2.7 Robustness

How much the developed method is robust? This parameter performs by conduct experiments as per ICH guidelines. Such values indicate that if a minor deliberate variation in the method parameter its capability to remain unaffected [25]. Performed analysis (n=3) in level -0.1, 0, +0.1 in parameters flow rate, pH and mobile phase composition.

2.8 System Suitability

Instrument performance analysis checks by design the experiment to calculate the precision value by injecting six replicate samples of the standard preparation. Important parameters like resolution, tailing factor, and theoretical plate number is measure.

3. RESULTS AND DISCUSSION

The optimized mobile phase composition at fixed pH and its flow rate were finalized by linearity, sensitivity, system suitability, selectivity, the lesser time required for analysis (low retention time), peak parameters. The mobile phase 25 mM potassium dihydrogen phosphate buffers: methanol (20:80), methodical chromatographic separation of ASP, ATO and CLO (16 µg mL^{-1}) with the retention time (min) of ASP- 1.85, ATO- 3.02, and CLO -6.9 min [Fig. 2].

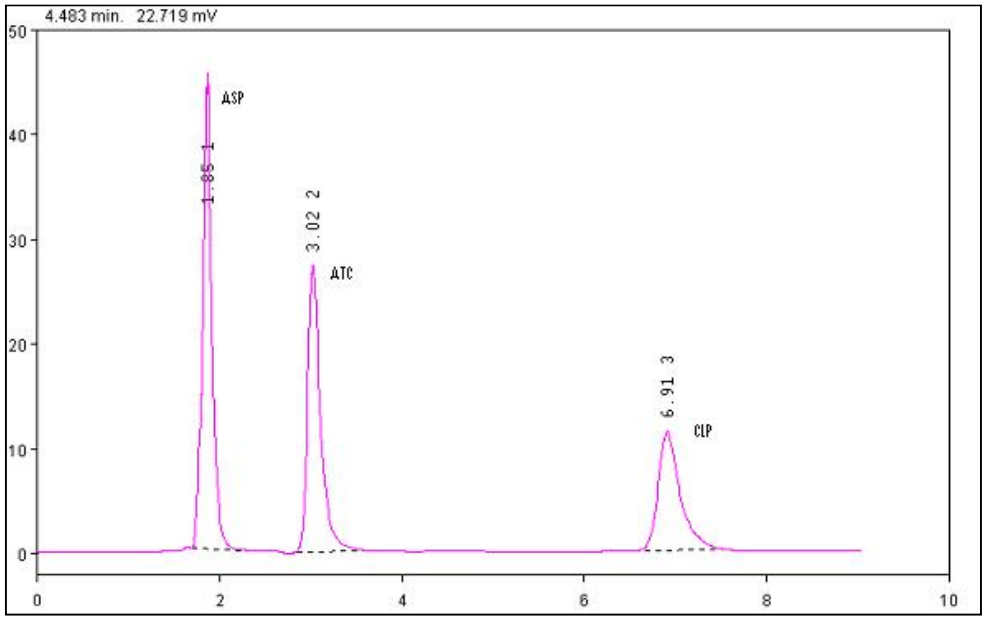

Fig. 2. Chromatogram of mixture of ASP, ATO and CLO in optimized conditions

3.1 Linearity and Range

The result of linearity and concentration range Table-1. Evaluated the value by performed analysis of working standard solutions of ASP, ATO, and CLO, seven different concentrations (0.5- 32 µg mL^{-1}). Regression analysis performed to find out the value of the calibration equation and correlation coefficient by injecting (n=3) for each seven concentration. Each drug was found linear in the concentration range.

3.2 Accuracy Precision and Recovery Studies

To find out the value of accuracy of the method by comparing the result of quality control samples with the result of the calibration standard curve. The value of standard deviation (SD) and percentage relative standard deviation (%RSD) found within the acceptable range. The precision of the method was established by carrying out an analysis of the analytes based on standard deviation and percentage relative standard deviation of intra-day and inter-day analysis. Intra and inter-day variability were determined by results from triplicate injections. Percentage recovery studies performed and result found ASP (98.84-100.11), ATO (97.76-99.99) and CLO (97.99-99.91) Table-1.

3.3 Selectivity and Specificity

Examination of interfering components in the mixture of drugs was studied, compared with the standard of the respective drugs and mobile phase chromatogram. A very clear chromatogram has a retention time (min) of ASP-1.85, ATO-3.02 and CLO-6.9. No variation in the retention time as well as interfering peaks at retention time and solvent-free from each peak [Fig.-3]. This show method selective and specific for the determination ASP, ATO, and CLO simultaneously.

3.4 Quantification of LOD & LOQ

Determination of limit of detection and quantification was performed (n=3) value in Table-1. The developed method indicates that it was very sensitive to quantify all the drugs.

3.5 Stability

Plotting the standard curve of the mixture sample on different days concerning quality control for determining the stability, the result analysis in terms for values of SD, %RSD and % bias for each drug in different days shown in Table-2.

3.6 Interaction Studies

As shown in [Table-3] revealed from the analysis that there is no interaction with the drugs at the retention time of individual drugs. Performed by comparing the peak area of individual drugs with the peak area of the same drug in the mixture at the same concentration, acceptable range ±20% in the peak area of individual drug versus peak area of the drug.

Table 1. Linearity regression, accuracy and precision

Parameter	ASP	ATO	CLO
Linearity range (µg ml^{-1})	0.5-32	0.5-32	0.5-32
r	0.989	0.993	0.994
r^2	0.979	0.988	0.989
Regression equation	y = 43.55x+3.528	y = 39.12x+1.885	y = 25.39x-0.978
LOD (µg/mL)	0.04,	0.04	0.08
LOQ (µg/mL)	0.14	0.13	0.28
Retention time	1.85	3.02	6.91
Accuracy (n=3)			
SD	0.32-0.58	0.42-0.68	0.52-0.48
RSD	0.48-0.31	0.58-0.31	0.38-0.32
Precision		**Intra-day (n=3)**	
SD	0.27-0.28	0.46-0.38	0.52-0.22
%RSD	0.26-0.27	0.56-0.87	0.53-0.32
		Intra-day (n=3)	
SD	0.33-0.38	0.56-0.87	0.62-0.72
%RSD	0.28-0.47	0.66-0.78	0.83-0.92

Table-2. Stability studies

Drug	Day	Clam (µg)	Obtn. conc. (µg)	SD	% RSD	% bias
ASP		0.75	0.76	0.15	0.47	1.50
	I	3	3.02	0.22	0.17	0.25
		24	23.91	1.36	0.13	-0.35
		0.75	0.74	0.17	0.55	1.96
	III	3	3.01	0.36	0.28	0.26
		24	23.85	4.38	0.43	-0.60
		0.75	0.76	0.18	0.62	1.73
	VII	3	2.87	1.01	0.88	-4.43
		24	23.41	1.24	0.12	-2.44
ATO		0.75	0.75	0.53	2.19	0.15
	I	3	2.99	0.06	0.06	-0.14
		24	23.96	0.81	0.11	-0.01
	III	0.75	0.73	0.48	2.07	2.57
		3	3.02	0.31	0.32	0.06
		24	23.99	0.87	0.11	--0.01
	VII	0.75	0.72	0.84	3.64	-2.76
		3	3.01	0.07	0.07	0.47
		24	24.01	0.92	0.11	0.05
CLO		0.75	0.75	0.06	0.38	0.95
	I	3	2.99	0.02	0.03	-0.03
		24	23.99	0.33	0.05	-0.03
	III	0.75	0.75	0.09	0.57	0.8
		3	3.01	0.36	0.51	0.5
		24	24.02	0.43	0.07	0.05
	VII	0.75	0.72	0.03	0.19	-1.03
		3	3.02	0.03	0.04	0.95
		24	24.01	0.25	0.04	0.04

Table-3. Interaction studies

Drugs	Drugs existence	Range (µg mL^{-1})	% peak area diff.	S.D.	% RSD
ASP	Individual	0.5-32	6.63 to5.70	0.64-0.72	2.90-0.05
	Mixture			0.06-1.9	0.33-0.14
ATO	Individual	0.5-32	11.01to14.35	0.53-3.68	2.78-0.29
	Mixture			0.09-2.57	0.57-0.22
CLO	Individual	0.5-32	1.75 to -2.09	0.07-1.22	0.60-0.15
	Mixture			0.17-1.87	1.45-0.22

3.7 Robustness and System Suitability

The developed method has robust checked by experimenting with different parameters and calculates the standard deviation and percentage relative deviation results in [Table-5]. The analytical procedure has been validated, from the system suitability test ensure that the resolution between different peaks of interest is good. Also, the value of each drug for other parameters find out by perform analysis met the acceptance criteria on all days [Table-5]. ASP, ATO, and CLO are eluted by forming symmetrical single peaks well separated from the solvent front [Fig. 2].

3.8 Application

3.8.1 Analysis of tablets

The validated method makes use of the test to ensure that the method was fit for a particular analytical purpose. For the tablet analysis, calibration curves were plotted for ASP, ATO and CLO at different concentration levels (0.25-32 µg ml^{-1}).

3.8.2 Analysis of the quality control sample

The quality control samples were analyzed at three concentration levels (n=3) as high (24 µg ml^{-1}), medium (3 µg ml^{-1}) and low (0.75 µg ml^{-1}).

Table 4. Analysis of quality control sample

	Linearity range	Conc. (µg mL^{-1})	Obtained conc. (µg mL^{-1})	SD	% RSD	% Bias
ASP		0.75	0.74	0.03	0.16	-0.73
(n=3)		3	2.94	0.03	0.25	-1.75
		24	24.05	0.41	0.04	0.02
ATO	(0.25-32 µg mL^{-1})	0.75	0.74	0.33	1.41	-0.69
(n=3)		3	3.02	0.29	0.29	0.71
		24	24.04	0.99	0.12	0.18
CLO		0.75	0.74	0.41	2.49	-0.01
(n=3)		3	3.02	0.27	0.37	0.69
		24	24.03	0.41	0.07	0.13

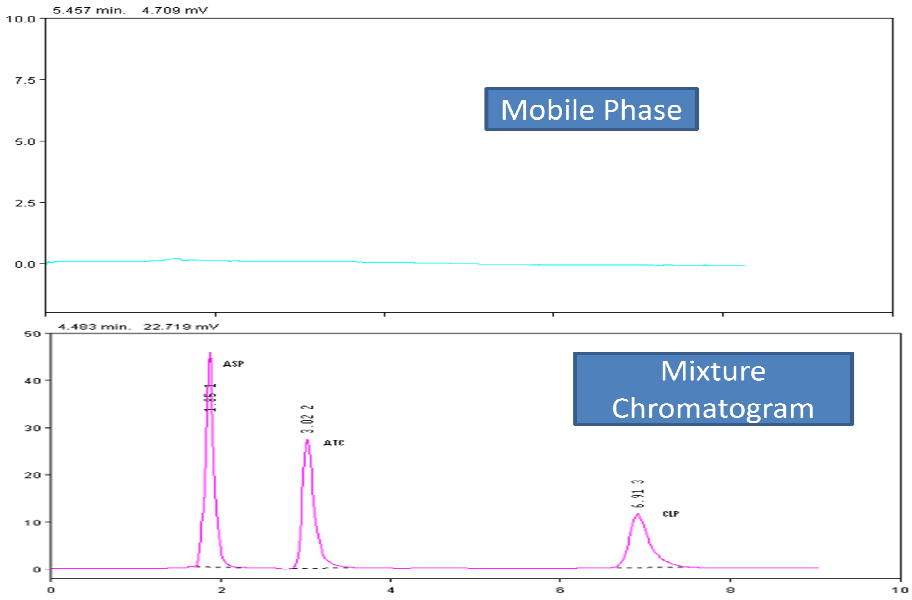

Fig. 3. Comparative Chromatogram of mobile phase and mixture of ASP, ATO and CLO

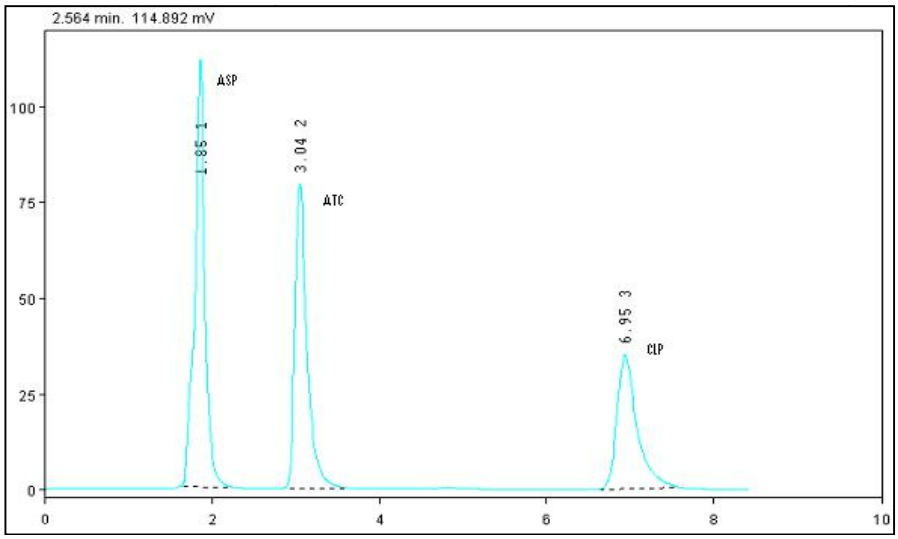

Fig. 4. Chromatogram of test sample containing ASP, ATO and CLO in tablet dosage form

Table-5. Robustness and system suitability

	ASP			ATO			CLO		
Parameter	**Flow rate**	**pH**	**Mobile phase composition**	**Flow rate**	**pH**	**Mobile phase composition**	**Flow rate**	**pH**	**Mobile phase composition**
SD	0.012	0.016	0.019	0.041	0.036	0.038	0.020	0.016	0.028
%RSD	0.65	0.87	0.75	1.35	1.19	1.125	0.28	0.23	0.27
System suitability									
Resolution	-			5.51			11.69		
Capacity factor (k')	0.85			2.04			5.95		
Theoretical plates	1674			2382			4398		
Asymmetry	0.800			2.000			1.964		

Table-6. Analysis of tablets

	Conc. (µg/ml)	**Individual**				**Mixture**				**Tablets (µg mL^{-1})**		
		Obt. Conc.(µg/ml)	**±SD**	**% CV**	**% Bias**	**Obt. conc.**	**±SD**	**% CV**	**% Bias**	**Qnt. claim**	**Qnt. found.**	**%**
ASP	0.75	0.77	0.44	1.51	3.49	0.74	0.36	1.29	-0.58			
(n=3)		3.09	0.94	0.77	3.27	2.90	1.05	0.879	-3.11	75	74.95	99.93
		23.92	3.43	0.35	-0.32	23.03	5.3	0.57	-4.06			
ATO	3	0.75	1.08	4.46	1.29	0.75	0.09	3.82	-0.45			
(n=3)		3.04	0.79	0.79	1.41	3.05	0.89	0.89	0.12	40	40.01	100.00
		23.93	0.29	0.29	-0.27	23.85	4.48	0.55	-0.62			
CLO		0.78	0.61	3.41	4.54	0.76	0.63	3.65	1.21			
(n=3)	24	3.07	0.63	0.86	2.42	3.06	0.3	0.41	2.01	75	74.90	99.87
		24.39	1.36	0.23	1.65	24.26	2.25	0.38	1.09			

The concentration of quality control samples was determined with the help of a linear equation obtained from the calibration curve. Result of the analysis [Table-4].

3.8.3 Analysis of test solution sample

Tablets were taken for testing and the aliquots were made, and samples were analyzed at the three concentrations which are high, medium and low. The triplicate injection of each concentration was given, and the average peak area was determined. The concentration of the test sample was determined with the help of a linear equation obtained from the calibration curve. Further, the percentage bias in area and concentration was calculated using the following formula:

$$\%\ bias = \frac{\text{observed concentration} - \text{claimed concentration x } 100}{\text{claimed concentration}}$$

The claimed concentration for ASP, ATO, and CLO was 75, 40 and 75 mg per tablet, respectively [Tablet-6].

4. CONCLUSION

This new method developed for simultaneous estimation of ASP, ATO, and CLO in the combined dosage form is very robust, specific, and precise. The chromatogram separation and peak shape symmetry are very good. Successfully validated the method as per the ICH guideline, almost all the parameters analyzed and results are in an acceptable range. Thus, the described method is acceptable for schedule analysis and quality control of the pharmaceutical dosage form.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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